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Development of a Virosome Vaccine for Newcastle Disease Virus

Darrell R. Kapczynski and Terrence M. Tumpey

Southeast Poultry Research Laboratory, Agricultural Research Service, USDA,
934 College Station Road, Athens, GA 30605

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SUMMARY. In an effort to protect chickens against Newcastle disease (ND), a nonreplicating virosome vaccine was produced by solubilization of Newcastle disease virus (NDV) with Triton X-100 followed by detergent removal with SM2 Bio-Beads. Biochemical analysis indicated that the NDV virosomes had similar characteristics as the parent virus and contained both the fusion and hemagglutinin-neuraminidase proteins. To target the respiratory tract, specific-pathogen-free chickens were immunized intranasally and intratracheally with the NDV virosome vaccine. This vaccine was compared with a standard NDV (LaSota) live-virus vaccine for commercial poultry. Seroconversion ($\geqslant$ four fold increase in hemagglutination inhibition [HI] antibody titers) was achieved in all birds vaccinated with the virosome vaccine. Upon lethal challenge with a velogenic NDV strain (Texas GB), all birds receiving either vaccination method were protected against death. Antibody levels against NDV, as determined by enzyme-linked immunosorbent assay and HI titer, were comparable with either vaccine and increased after virus challenge. These results demonstrate the potential of virosomes as an effective tool for ND vaccination.

RESUMEN. Desarrollo de una vacuna virosomal para el virus de la enfermedad de Newcastle.

En un intento para proteger a los pollos contra la enfermedad de Newcastle, se produjo una vacuna virosomal no replicativa mediante la solubilización del virus de la enfermedad de Newcastle con Tritón X-100 seguido por la remoción del detergente con SM2 Bio-Beads. Los análisis bioquímicos indicaron que los virosomas del virus de la enfermedad de Newcastle mostraron características similares al virus original presentando igualmente las proteínas de fusión y hemoaglutinina-neuraminidasa. Con el objetivo de restringir el virus al tracto respiratorio se inmunizaron pollos libres de patógenos específicos por vía intranasal e intraocular con la vacuna virosomal del virus de la enfermedad de Newcastle. Se comparó la vacuna virosomal con una vacuna comercial estándar a virus vivo del virus de la enfermedad de Newcastle (cepa LaSota). Se logró seroconversión (definida como el incremento en por lo menos cuatro veces de los títulos de anticuerpos inhibidores de la hemoaglutinación) en todas las aves vacunadas con la vacuna virosomal. Al ser desafiadas con una capa velogénica letal del virus de la enfermedad de Newcastle (Texas GB), la totalidad de las aves que recibieron alguna de las dos vacunas fueron protegidas contra la mortalidad. Los niveles de anticuerpos contra el virus de la enfermedad de Newcastle, obtenidos mediante la técnica de inmunoensayo con enzimas asociadas (ELISA) e inhibición de la hemoaglutinación, en los grupos que recibieron la vacuna comercial o la vacuna virosomal fueron comparables, mostrando un incremento después del desafío. Se demuestra el potencial de los virosomas como una herramienta efectiva para la vacunación contra la enfermedad de Newcastle.

Key words: avian paramyxovirus, veterinary virology, virosome vaccine, chickens, immunology

Abbreviations: C = challenged; DMEM = Dulbecco minimal essential medium; ELD_{50} = 50% embryo lethal dose; ELISA = enzyme-linked immunosorbent assay; F = fusion; GFP = green fluorescent protein; HA = hemagglutination; HI = hemagglutination inhibition; HN = hemagglutinin-neuraminidase; L = RNA-dependent RNA polymerase; M = matrix; MHC = major histocompatibility complex; NC = nonchallenged; ND = Newcastle disease; NDV = Newcastle disease virus; NP = nucleoprotein; P = phosphoprotein; PBS = phosphate-buffered saline; PBS-T = 0.05% Tween 20 in phosphate-buffered saline; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPF = specific-pathogen free

Newcastle disease virus (NDV) is a member of the Paramyxoviridae family and is responsible for a highly infectious viral disease of poultry (1). The virus is enveloped and contains a negative-sense, single-stranded RNA genome that codes for six proteins including an RNA-dependent RNA polymerase (L), fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, matrix (M) protein, phosphoprotein (P), and nucleoprotein (NP) (11). The F protein is responsible for fusion with host cells and is cleaved by cellular proteases into a disulfide linked F1-F2 peptide (14,19,30). The HN protein binds to sialic acid–containing molecules found on cell surfaces and has demonstrated neuraminidase activity (1). The F and HN transmembrane glycoproteins are the principal antigens that elicit a protective immune response (1). Monoclonal antibodies directed against these glycoproteins neutralize NDV by inhibiting cell fusion and attachment (28).

Clinically, NDV isolates can be categorized into one of three main pathotypes (1). Lentogenic strains have low virulence, cause mild or unapparent respiratory or enteric infections, and are utilized as live-virus vaccines. Mesogenic isolates of NDV mainly cause respiratory and nervous signs but not high mortality. Highly virulent NDV isolates are termed velogenic, and infection can cause high mortality in chickens. Velogens can be further divided as neurotropic or viscerotropic depending on the clinical signs and lesions associated with infection.

Many different types of vaccines have been produced for protection against NDV outbreaks. Protection against Newcastle disease (ND) reportedly involves both humoral and cellular immunity (28,38,39). Inactivated oil-emulsion vaccines are generally used for individual injection of birds, which is both laborious and costly. Low-virulence live-virus vaccines have been produced and are usually administered to drinking water or sprayed by aerosol droplet (1,6,7). Subunit, recombinant, and DNA vaccines have also been developed and provide various degrees of protection against ND (8,9,15,24,31,40).

Virosome vaccines were first described in 1975 for the combination of influenza subunits and liposomes that resembled the original shape of the virus (2). Containing viral membrane proteins within a liposome complex, these virosome vaccines are noninfectious because they do not possess genetic nucleic acid. Live-virus vaccines have a disadvantage of causing respiratory disease under certain field conditions, whereas inactivated vaccines require costly individual injection. Virosome vaccines may be advantageous because they are as safe as inactivated vaccines, but they retain the ability to attach and fuse with host cells as live virus. Virosome vaccines have been described for influenza (13,18), rabies virus (21), hepatitis A virus (33), herpes simplex virus (23), and Sendai virus (29). In addition to stimulating humoral responses, these vaccines can deliver antigen to endosomes, resulting in antigen presentation by major histocompatibility complex (MHC) class I, and stimulate cytotoxic T lymphocytes (4,47).

In the present study, we described the morphologic and immunologic characteristics of a NDV virosome vaccine and examined protective immunity in chickens after intranasal vaccination with subsequent lethal NDV challenge.

MATERIALS AND METHODS

Chickens. We obtained one-day-of-age, mixed sex, specific-pathogen-free (SPF) white Plymouth Rock chickens from our flock at the Southeast Poultry Research Laboratory. Birds were maintained in Horsfall isolation units located in the biosafety level 3 agriculture facility, with feed and water *ad libitum.*

Viruses and cell culture. NDV strains LaSota, a lentogenic field isolate that was subsequently used as a ND vaccine, and Texas GB, a neurotropic velogenic isolate, used as vaccine and challenge virus, respectively, were from the laboratory repository (1). NDV was propagated and titrated in 9-to-11-day-old SPF chicken embryos via the chorioallantoic sac route. The allantoic fluid was collected and centrifuged at 3000 $\times$ *g* for 30 min. NDV in allantoic fluid was sucrose purified as previously described (39). Vero cells were obtained from the American Type Culture Collection and used for fusion experiments. Cells were maintained in Dulbecco minimal essential medium (DMEM) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 mg/l streptomycin. Cells were grown to 60%–70% confluency on 18-mm round glass coverslips at 37 C in 5% CO_2.

Preparation of virosomes. NDV virosomes were produced as previously described for Sendai virus with minor modifications (5). Briefly, sucrose-purified NDV LaSota was resuspended in phosphate-buffered saline (PBS), pH 7.2, at a protein concentration of 5 mg/ml. Triton X-100 (Fisher Scientific, Atlanta, GA) was added to a final concentration of 2% (v/v) and incubated at room temperature for 1 hr with gentle shaking. The suspension was centrifuged for 1 hr at 100,000 $\times$ *g* to remove nucleocapsid complexes. Detergent was removed by the stepwise addition of methanol-washed SM2 Bio-Beads (Bio-Rad Laboratories, Inc., Hercules, CA) (16,25). Briefly, 160 mg of SM2 Bio-Beads was

added to 2 ml of supernatant, gently mixed by inversion, and allowed to incubate for 2 hr at room temperature, followed by the addition of 320 mg of SM2 Bio-Beads for an additional 2 hr at 4 C and an additional 640 mg overnight at 4 C. The reconstituted NDV virosomes were collected with a needle and syringe to exclude the SM2 Bio-Beads. To ensure vaccine inactivation, reconstituted virosomes (diluted 1:10 in PBS) were tested by inoculation into 9-to-11-day-old SPF chicken embryos as above and monitored for mortality. Allantoic fluid was harvested, passaged three times, and tested for hemagglutination (HA) activity. All virosome vaccine preparations were negative for NDV. A commercial kit was used to determine protein concentration (Pierce, Rockford, IL).

Immunoelectron microscopy. A formvar-carbon–coated 400-mesh copper grid was incubated with NDV antiserum in a wet chamber 30 min. The excess antiserum was drawn off with filter paper, and the grid was incubated with clarified NDV or virosome sample for 30 min. Excess sample on the grid was drawn off with filter paper and rinsed with 1–2 drops of deionized water. After the excess water was blotted off, the grid was placed on a drop of 2% aqueous phosphotungstic acid, pH 7.0, for 30 sec. After excess stain was drained, the grid was allowed to dry on filter paper before it was viewed with the JEM-1210 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at an operating voltage of 120 kV.

Fusion studies. To examine fusogenic properties of NDV LaSota virosomes, we incorporated the reporter plasmid pEGFP-N1 (CLONTECH Laboratories, Inc., Heidelberg, Germany), encoding for green fluorescent protein, into the virosomes during reconstitution. Briefly, Triton X-100 solubilized LaSota envelope proteins were added to 10 μg pEGFP-N1 (previously condensed with poly-L lysine; 1:1.5 [w/w]) and reconstituted by the stepwise addition of SM2 Bio-Beads. Unincorporated DNA was removed by DNase I treatment as previously described (34). Purified virosomes containing pEGFP-N1 were inoculated onto Vero cells in the presence of exogenous trypsin (0.5 μg/ml) for 1 hr. The medium was removed and cells were washed 3× in DMEM. After 48–72 hr, cells were examined for GFP expression under an Olympus BK41 (Olympus America Inc., Melville, NY) fluorescent microscope. For comparison, Vero cells were transfected with either 1 μg pEGFP-N1 with the use of lipofectamin (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations or lipofectamin alone.

Immunization and virus challenge. Seventy-two 1-day-old SPF chickens were arbitrarily separated into three groups of 24 birds. Birds in group 1 received 100 μl of PBS, pH 7.4, via intranasal (50 μl) and intratracheal (50 μl) routes at 1 and 14 days of age. Birds in group 2 received live-virus LaSota vaccine at 1×10^6 50% embryo infectious dose/bird via intranasal and intratracheal routes as described above. Birds in group 3 received 100 μl of NDV LaSota virosomes (10 μg/bird) as described above. Two weeks after the second vaccination (day 28), one-half of the birds in each group were challenged intramuscularly with Texas GB (10^2 50% embryo lethal dose [ELD_{50}]/bird). Unchallenged birds were given 100 μl PBS via intramuscular injection. After challenge, birds were monitored daily for clinical signs of disease (muscular tremors, torticollis, and paralysis of wings and legs) and mortality. Birds that displayed severe clinical signs of disease were euthanized by overdose of sodium pentobarbital. Serum samples were taken by wing bleed at 3, 7, and 14 days postchallenge.

NDV enzyme-linked immunosorbent assay (ELISA). A commercial ELISA test kit (Flockcheck™; IDEXX Laboratories, Inc., Westbrook, ME) was used to test serum for antibodies against NDV. Chicken serum samples were diluted 1:500 and incubated in 96-well microtiter plates containing NDV antigen. The ELISA was performed according to the manufacturer's recommendations.

HA and hemagglutination-inhibition (HI) assays. The HA and HI tests were performed by standard microtiter plate methods. The HI tests were performed as previously described, with 10 HA units per well (43)

Western blot. Purified NDV proteins from whole virus and virosome preparations were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel by the Bio-Rad Criterion system (22). The separated proteins were transferred onto a 0.45 μM nitrocellulose membrane (Trans-Blot transfer membrane, Bio-Rad) by semidry blot with a Bio-Rad Trans-Blot SD Semi-Dry Cell (45). Transfer was performed at a constant voltage of 15 V for 20 min. After transfer, unbound protein binding sites were blocked by incubation with 5% skim milk in PBS-T (0.05% Tween 20 in PBS) for 1 hr at room temperature. Anti-NDV antisera were diluted 1:1000 in blocking buffer and added to the membrane for 1 hr. The primary antibody was removed by three washes in PBS-T, and the membrane was reacted with secondary antibody (horseradish peroxidase–labeled goat anti-chicken immunoglobulin G; Southern Biotech Associates, Inc., Birmingham, AL) at a 1:2000 dilution for 1 hr. After washing, the membrane was reacted with ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's recommendations and exposed to Hyperfilm ECL (Amersham Biosciences). The film was developed with Kodak GBX developing reagents (Eastman Kodak Co., Rochester, NY) according to the manufacturer's recommendations.

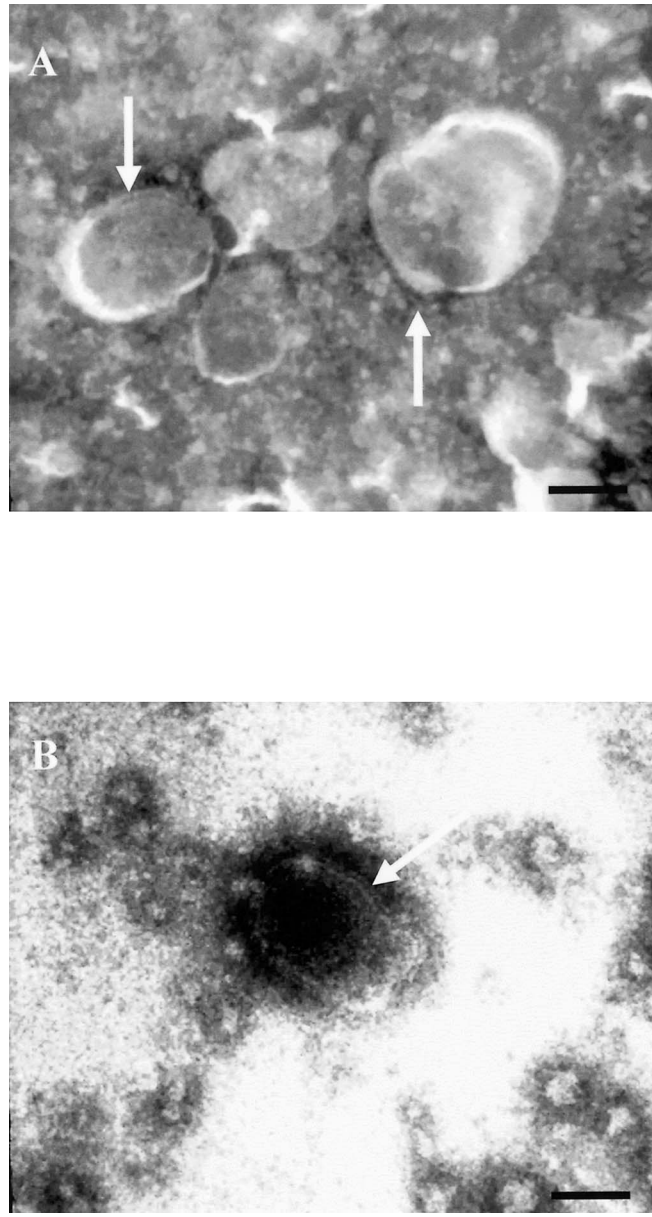

Fig. 1. Electron micrograph comparison of LaSota NDV (A) with NDV virosome vaccine (B). Bar = 100 nm. 100,000×.

RESULTS

Morphologic examination of NDV virosome vaccine. Preparations of NDV virosomes were compared for size, shape, and structural integrity with live-virus NDV by electron microscopy. As shown in Fig. 1A, the NDV virosome vaccine forms unilamellar liposomes with spiked projections (arrow) on the outer surface. These resemble spikes also found on LaSota live virus (arrow) in Fig. 1B. Both live virus and virosomes exhibited rough spherical shapes approximately 75–200 nm in diameter.

Characterization of virosome vaccine.

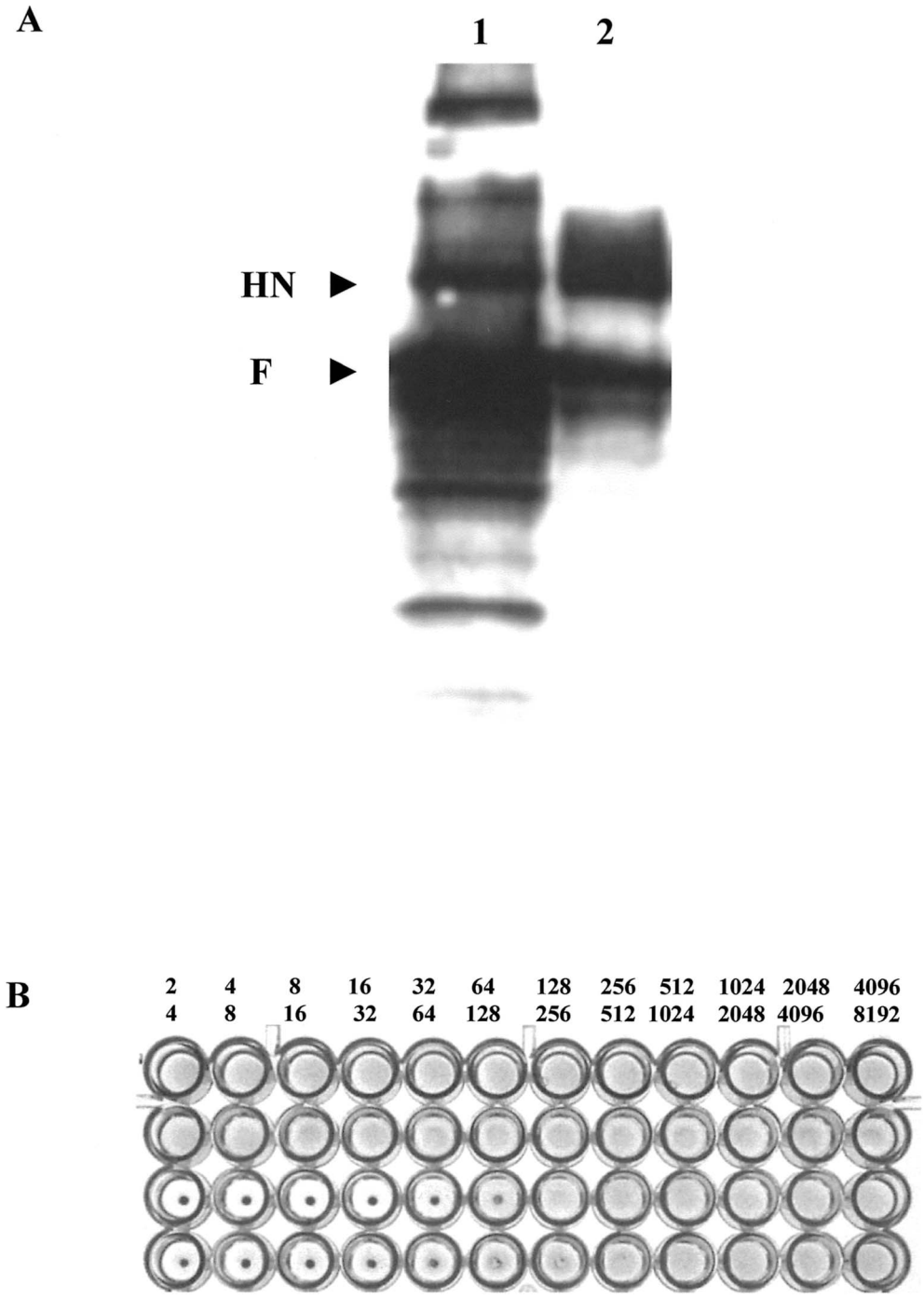

Fig. 2. Biochemical and functional analysis of NDV virosomes. (A) Western blot of NDV LaSota (1) and NDV virosomes (2) demonstrating F and HN proteins contained in vaccine. (B) HA (top dilution series) and HI (bottom dilution series) titration of NDV LaSota (1 and 3, respectively) and NDV virosomes (2 and 4, respectively).

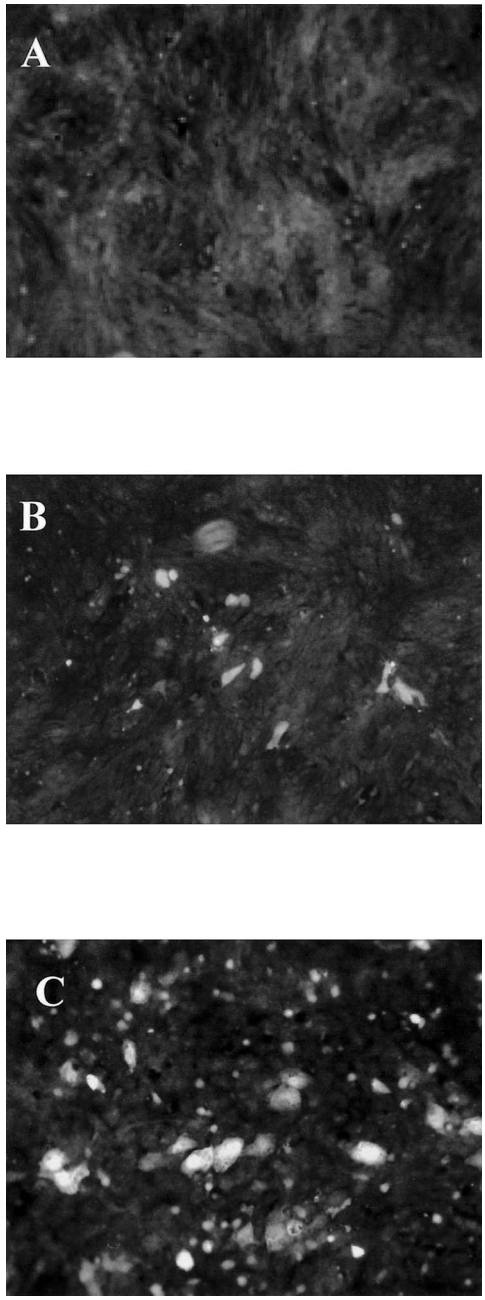

Fig. 3. Green fluorescent protein (GFP) expression after transfection of Vero cells with pEGFP-N1 with lipofectamin or pEGFP-N1–loaded NDV virosomes demonstrating fusion properties of NDV virosomes. (A) Vero cell negative control. (B) Vero cells transfected with 1 μg pEGFP-N1 with lipofectamin. (C) pEGFP-N1–loaded NDV virosomes inoculated on Vero cells in the presence of trypsin.

The protein composition of the NDV virosome vaccine was determined by SDS-PAGE in the presence of β-mercaptoethanol followed by western blot analysis with NDV antiserum. Positive reaction of protein bands corresponding to F and HN glycoproteins of NDV were observed in virosome preparations when compared with intact virus (Fig. 2A). In some preparations, a reaction was also observed with a 40-kD protein (data not shown).

To ensure virosome preparation did not interfere with sialic acid binding or antigenic determinants of the HN protein, HA and HI tests were compared between live virus and virosome vaccine. As demonstrated in Fig. 2B, both live virus and virosomes were capable of hemagglutinating chicken red blood cells. Preincubation of virosomes with anti-NDV antisera inhibited HA to titers observed with the live virus.

Expression of pEGFP-N1 in Vero cells. To examine whether the fusion protein contained within the virosomes remained functional after production, we encapsulated a plasmid construct containing the gene encoding the green fluorescent protein (GFP) within the NDV virosomes. Fig. 3A represents control Vero cells that received lipofectamin alone. As illustrated in Fig. 3B, transfection of Vero cell monolayers with virosomes containing pEGFP-N1 resulted in GFP expression confirming functional fusion. Although we did not seek to optimize encapsulation or transfection with DNA-loaded virosomes, plasmid DNA coupled with lipofectamin appeared to have higher transfection rates than virosome-derived plasmid DNA on the basis of GFP expression (Fig. 3C).

Virosome vaccination serology and challenge with Texas GB. Nonvaccinated, nonchallenged (NC) birds (PBS-NC) lacked antibodies against NDV (Table 1). In contrast, groups of nonchallenged birds that received either NDV live virus (LaSota-NC) or virosomes (Virosomes-NC) both had ELISA and HI antibody titers. Although the LaSota live virus consistently produced higher HI responses compared with birds that received virosomes, the increased HI response was not unexpected because replicating vaccine virus may be expected to produce a higher antigen load of the NDV HN than the virosome vaccine. The increased HI response was also observed when comparing LaSota-vaccinated, Texas GB–challenged (C) groups (LaSota-C) with virosome-vaccinated, Texas GB–challenged groups (Virosomes-C).

Control birds that received PBS did not survive challenge and exhibited 100% mortality by day 7

Table 1. Serum antibody response after intranasal vaccination with LaSota live virus or NDV virosomes (at 1 and 14 days of age) and challenge (at 28 days of age) with velogenic Texas GB.

Group[A]	*n*	Test	Postchallenge sample		
			Day 3	Day 7	Day 14
PBS-NC	12	ELISA[B]	0	0	0
		HI[C]	1	2	2
LaSota-NC	12	ELISA	5757 (2424)	6254 (2147)	5576 (1321)
		HI	10	11	8
Virosomes-NC	12	ELISA	7659 (829)	3485 (2829)	2639 (640)
		HI	9	7	7
PBS-C	12	ELISA	0	NA[D]	NA
		HI	1.5	NA	NA
LaSota-C	12	ELISA	3137 (918)	8430 (2783)	5761 (2436)
		HI	9	13	13
Virosomes-C	12	ELISA	5995 (2383)	10,989 (1360)	1943 (497)
		HI	6	8	10

[A]NC = non challenged; C = challenged (intramuscularly with 100 ELD_{50} Texas GB NDV).
[B]Results are expressed as mean (standard error of the mean in parentheses). Titers >396 are considered positive.
[C]Geometric mean titer expressed as reciprocal $\log_2$.
[D]NA = not applicable.

postchallenge (Fig. 4). Birds that received either NDV virosomes or live virus were 100% protected from challenge throughout the course of the experiment, indicating protective immunity was provided by both vaccines tested. All birds in nonchallenged groups remained healthy throughout the experiment.

DISCUSSION

The goal of this study was to produce a NDV virosome vaccine and investigate immunogenicity and protective immunity to determine if virosomes have an advantage over the standard live NDV vaccine in chickens. The NDV virosomes provided complete protection from lethal challenge whereas nonvaccinated birds developed disease and died. As expected, the standard live NDV vaccine provided protection against virulent challenge virus. Previous reports have detailed the production of virosomes from enveloped viruses for studies involving fusion proteins, gene transfer, and vaccination (5,10,18,29, 32,33,34,35). Ours is the first report to describe protective immunity after vaccination with NDV virosomes.

In the present study, evaluation of NDV virosomes revealed a unilamellar structure similar in size and confirmation to the intact virus. Two major protein bands, corresponding to the F and HN glycoproteins on the basis of molecular weight and functional analysis, were detected by western blot analysis. These results are in agreement with the recent work of Cobaleda *et al.* (10), which identified the F and HN glycoproteins from reconstituted NDV liposomes. The minor protein band observed at approximately 40 kD is believed to be residual M protein because this protein associates with the viral surface glycoproteins from the inner surface of the viral envelope (12). Functionally, the NDV virosome vaccine retained both HA and fusion biologic properties that would mimic NDV live-virus vaccine *in vivo.* The orientation of the surface proteins on the virosomes appears to resemble that found on the virus particle, which would be important for receptor binding and antigen presentation.

Comparison of the antibody responses after virosome vaccination resulted in increases of both ELISA and HI antibody titers that were comparable with the live virus–vaccinated chickens. An association between the presence of ELISA or HI antibodies and protection from challenge was observed during the experiment. Previous research with virosomes of influenza, hepatitis virus, and respiratory syncytial virus demonstrated both intranasal (13,27) and intramuscular (41) routes of vaccination increased humoral immune responses resulting in protection from challenge. In this study, two doses of virosomes were given per bird within a 2-wk interval. Whether two vaccinations is a requirement for protective immunity is the subject of ongoing studies.

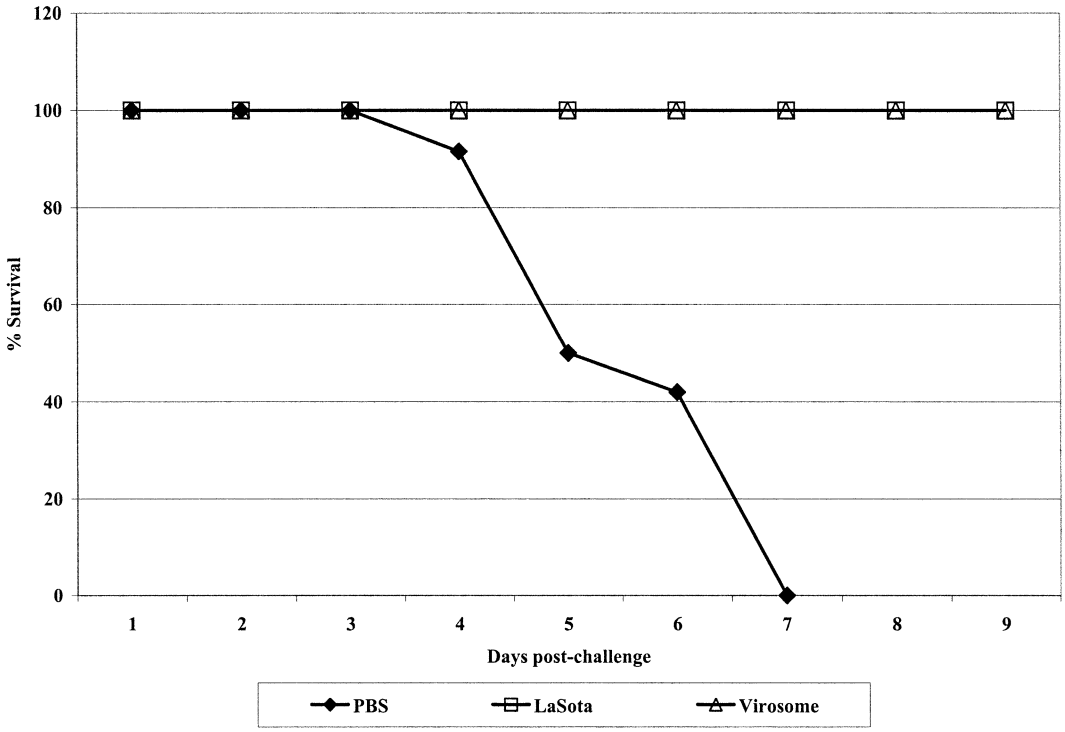

Fig. 4. Survival against lethal NDV challenge. Birds received intranasal vaccination with either LaSota live virus or NDV virosomes at days 1 and 14 of age. At day 28, birds were challenged intramuscularly with velogenic Texas GB.

One advantage of virosome vaccines is their nonreplicating properties. Aside from safety, virosomes do not contain antigens from the replication machinery (e.g., L, P, or NP proteins). Testing for antibodies to the NDV L, P, or NP proteins can identify birds that received exposure to replicating NDV and distinguish them from virosome-vaccinated birds. This procedure allows for screening to determine if field viruses are present and circulating within a poultry flock. Use of either live virus or inactivated virus vaccines would not allow this type of differentiation to be made because vaccinated birds would have antibodies to all NDV proteins.

Numerous investigators have identified the efficacy of inactivated NDV vaccines (17,26,42). Despite their safety and efficiency, inactivated vaccines are, in general, poor inducers of cellular immunity (20,44,46). The intention for the use of inactivated vaccines is to induce a humoral response that can effectively neutralize virus infectivity and replication. Virosomes have been determined to stimulate both MHC I and II pathways, thereby inducing humoral and cellular immune responses (3,4,37). Because NDV virosomes can bind and fuse with cells containing sialic acid residues, internalization of NDV antigens may also provide for MHC class I and II presentation and thereby stimulate virus-specific cytotoxic T cells. Cytotoxic T-cell responses were stimulated after virosome delivery of influenza (18) and Ebola virus antigen (36,37). Thus immunologically, virosomes act like an intracellular virus in this regard.

In conclusion, we have shown NDV virosomes delivered to the respiratory tract provide protection from lethal NDV challenge. This technique for vaccine production may prove useful for other enveloped avian viruses and has potential for intracellular delivery of macromolecules *in vivo* or *in ovo.*

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